DIRECT INCORPORATION OF HYDROXYPROLINE INTO PROTEIN OF SYCAMORE CELLS INCUBATED AT GROWTH-INHIBITORY LEVELS OF HYDROXYPROLINE

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Free hydroxyproline inhibits the growth of animals[1, 2] and plants.[3–7] The mechanism of this inhibition is not known. Among the theoretically possible effects of hydroxyproline are: (1) reduction of protein synthesis by lowering proline incorporation, (2) interference with proline hydroxylation, and (3) direct incorporation of hydroxyproline resulting in nonfunctional proteins.

Hydroxyproline residues in collagen arise from an hydroxylation of peptide-bound proline[8] (although data suggesting a very slight direct incorporation of hydroxyproline into chick embryo tissues have been reported).[9] A similar origin has been proposed for the hydroxyproline present in plant cell-wall protein.[10, 11] Thus any direct incorporation of hydroxyproline might result in nonfunctional proteins. Hitherto the major difficulty in demonstrating a direct incorporation of hydroxyproline arose from the alternative possibility of an indirect incorporation, through conversion of hydroxyproline to proline, followed by incorporation into protein and subsequent hydroxylation. This possibility can now be eliminated by the use of $\alpha\alpha'$-dipyridyl, a recently discovered inhibitor of proline hydroxylation.[12]

Materials and Methods.—A 10-ml portion taken from suspension cultures of exponentially growing sycamore cells (*Acer pseudoplatanus*) was used for each treatment.[13] The cultures were grown in a 20 per cent coconut-milk medium[14] with constant agitation by a gyratory shaker. After treatment with or without various inhibitors as described (Tables 1, 2, and 3), the cells were drained over a sintered-glass filter, rinsed with distilled water, disrupted by sonication, and separated by low-speed centrifugation into pellet and supernatant fractions. The cell-wall fraction was obtained from the pellet by extensive washing with 1 *M* NaCl, containing 200 μg/ml of L-proline and of L-hydroxyproline, followed by water washings. After each washing the cell-wall fragments were resedimented by a low-speed, one-minute centrifugation. Finally the pellet was suspended in water and portions taken for dry-weight determination and for hydrolysis (6 *N* HCl, 105°, 36 hr).

The supernatant fraction from the sonicated cells was made to 10 per cent trichloroacetic acid (TCA) and the precipitate centrifuged down after one hour at 2°. The pellet was dissolved in 0.5 *N* NaOH containing 200 μg/ml of L-proline and of L-hydroxyproline. Sufficient TCA was added to reprecipitate the protein which was again collected by centrifugation, twice washed with 5 per cent TCA, and then dissolved in 2 *N* NH_4OH. Portions were taken for quantitative assay[15] and for hydrolysis. All hydrolysates were applied to Whatman no. 4 paper and separated electrophoretically at pH 1.9.[16] Labeled amino acids, detected by radioautography, were eluted off the paper, and their radioactivity was determined by liquid scintillation counting. Portions of the filtered incubation media, collected earlier, were cleared by centrifugation and also counted.

Results and Discussion.—Sycamore cells incubated either with proline-C^{14} or

hydroxyproline-H^3 in the absence of any inhibitors (Table 1, treatments *A* and *D*) had similar ratios of labeled hydroxyproline to labeled proline in both their cytoplasmic and cell-wall proteins. The labeling patterns from proline-C^{14} and hydroxyproline-H^3 are similar also in the *Avena* coleoptile.[17] With $\alpha\alpha'$-dipyridyl present (Table 1, treatments *B* and *E*), the amount of radioactive hydroxyproline became very small (< 0.1% of control levels). These results show that $\alpha\alpha'$-dipyridyl totally inhibited proline hydroxylation in sycamore; yet it did not decrease the incorporation of radioactivity. Thus one can infer that all of the labeled protein-bound hydroxyproline present in cells after incubation with hydroxyproline-H^3 (Table 1, treatment *D*) must have been incorporated as proline which was subsequently hydroxylated.

TABLE 1

INCORPORATION OF LABELED PROLINE AND HYDROXYPROLINE INTO CYTOPLASMIC AND CELL-WALL PROTEINS

Treatment	Tracer	Inhibitors	Growth Δg, dry wt (% control)	Depletion of tracer from medium (%)	Radioactivity: Cytoplasmic Protein Cpm/mg ($\times 10^{-1}$)	Cytoplasmic Protein Hypro/proline	Cell Wall Cpm/mg ($\times 10^{-1}$)	Cell Wall Hypro/proline
A	Proline-C^{14}	None	100	95	6,380	0.07	3,850	6.0
B	Proline-C^{14}	Dipyridyl	40	92	7,920	0.005	3,800	0.005
C	Proline-C^{14}	Hypro*	32	95	8,540	0.08	5,000	6.2
D	Hypro-H^3	None	100	59	18,100	0.05	10,650	5.0
E	Hypro-H^3	Dipyridyl	40	59	25,000	0.004	10,800	0.003
F	Hypro-H^3	Hypro	30	96	3,540	4.7	1,150	16.0
G	Hypro-H^3	Dipyridyl + Hypro	32	96	3,000	4.7	700	8.5

After a 1-hr incubation with or without $\alpha\alpha'$-dipyridyl (0.2 mM) as specified, 1 μc of L-proline-C^{14} (200 μc/μmole) or 12.4 μc of L-4-hydroxyproline-5-H^3 (187 μc/μmole) was added with or without L-hydroxyproline (0.2 mM), as specified. The treatments were terminated 23 hr later. Results represent averages of duplicate experiments.

* Hydroxyproline is abbreviated as "hypro."

Treatment of cell cultures with growth-inhibitory amounts of L-hydroxyproline slightly enhanced proline-C^{14} incorporation, but did not affect hydroxylation of the incorporated proline (Table 1, treatment *C*). Thus, in sycamore, growth inhibition by hydroxyproline is not accompanied by a lowering of proline incorporation nor by any change in the degree of proline hydroxylation over the 24-hour period studied. Growth inhibition is accompanied, however, by an incorporation of hydroxyproline as shown in the treatments using tritiated hydroxyproline at growth-inhibitory concentrations (Table 1, treatments *F* and *G*). Compared to those of the control treatment (*D*), the hydroxyproline-H^3/proline-H^3 ratios in these treatments are two- to threefold higher in cell-wall protein and almost 100-fold higher in cytoplasmic protein.[18] Inclusion of $\alpha\alpha'$-dipyridyl along with the hydroxyproline resulted in a reduction by one half of the cell-wall hydroxyproline/proline ratio, but had no effect on the ratio for cytoplasmic protein. Presumably, $\alpha\alpha'$-dipyridyl prevented hydroxylation of the proline incorporated into cell-wall protein and thereby lowered the hydroxyproline/proline ratio, but did not affect the ratio for cytoplasmic protein because the proline in this fraction is not normally hydroxylated after incorporation.

Evidence indicating covalent bonding of the labeled hydroxyproline present in the cell-wall and cytoplasmic fractions comes from the following experimental results: (1) no detectable radioactivity was recovered in these two fractions from untreated

cells sonicated in the presence of hydroxyproline-H^3 or proline-C^{14}; (2) dialysis of the dissolved cytoplasmic protein fractions from treatments *F* and *G* (Table 1) resulted in no loss of radioactivity. Bonding of hydroxyproline-H^3 to proteins rather than to other macromolecules is suggested from experiments using cycloheximide, a potent inhibitor of protein synthesis at the ribosomal level.[19] In the presence of cycloheximide the incorporation of hydroxyproline-H^3 into cell-wall and cytoplasmic fractions was even more markedly reduced than that of proline-C^{14} (Table 2). Directly contrasting with sycamore is *Streptomyces antibioticus* in which the

TABLE 2

INHIBITION BY CYCLOHEXIMIDE OF AMINO ACID INCORPORATION

Tracer*	Cyclo-heximide (μg/ml)	Depletion of tracer from medium (%)	Radioactivity: Cytoplasmic Protein Cpm/mg (× 10^{-1})	Cytoplasmic Protein Control (%)	Cell Wall Cpm/mg (× 10^{-1})	Cell Wall Control (%)
Proline-C^{14}	0	92	16,950	100	35,000	100
Proline-C^{14}	1	92	6,230	37	5,070	15
Hypro-H^3	0	95	2,120	100	3,630	100
Hypro-H^3	1	94	301	14	334	9

After an hour's incubation with $\alpha\alpha'$-dipyridyl (0.2 mM) with or without cycloheximide as specified, tracer was added together with L-hydroxyproline (0.2 mM). Treatments terminated 23 hr later. Data averaged from results of duplicate samples.

* Tracer levels and specific activities same as specified in Table 1.

TABLE 3

EFFECT OF ADDING PROLINE WITH GROWTH-INHIBITORY AMOUNTS OF HYDROXYPROLINE ON GROWTH AND HYDROXYPROLINE INCORPORATION

Proline (mM)	Growth Δg, dry wt (% control)*	Depletion of tracer from medium (%)	Radioactivity: Cytoplasmic Protein Cpm/mg (× 10^{-1})	Cytoplasmic Protein Hypro/proline	Cell Wall Cpm/mg (× 10^{-1})	Cell Wall Hypro/proline
0.000	34	95	2860	3.2	6850	3.2
0.050	100	95	1085	1.7	1600	1.1
0.025	90	96	1510	3.2	2570	1.4

After an hour's incubation in the presence of 0.2 mM $\alpha\alpha'$-dipyridyl, 12.4 μc of L-hydroxyproline-5-H^3 was added together with unlabeled hydroxyproline (to 0.2 mM) and proline, as specified. Experiment terminated 23 hr later.

* Growth measurements were performed simultaneously with sycamore cells grown in the absence of $\alpha\alpha'$-dipyridyl, since growth is inhibited at 0.2 mM $\alpha\alpha'$-dipyridyl. Control samples grown without added hydroxyproline and proline.

synthesis of an hydroxyproline-containing actinomycin was enhanced by two inhibitors of protein synthesis (chloramphenicol and puromycin).[20] Since hydroxyproline is readily incorporated into actinomycin I of *S. antibioticus*,[21] we would expect an enhanced incorporation of hydroxyproline under conditions where protein synthesis is inhibited. Therefore, the marked inhibition of hydroxyproline incorporation by cycloheximide in sycamore indicates that the hydroxyproline-containing proteins present in sycamore after incubation at high hydroxyproline levels were synthesized on the ribosomes.

The activation of hydroxyproline, a step necessary for its incorporation into protein, may be catalyzed by a prolyl-RNA synthetase. A much lower affinity of this enzyme for hydroxyproline than for proline would explain why hydroxyproline was incorporated only when it was present at a high concentration. Frazer[22] found that rat-liver prolyl-RNA synthetase activated small amounts of hydroxyproline, especially when the hydroxyproline concentration was high (4.0 mM). Indirect evidence for a synthetase with similar affinities in plant systems comes

from observations that addition of proline in amounts much lower than that of the hydroxyproline eliminated the growth-inhibitory effect of hydroxyproline.[6, 7] The addition of proline to sycamore cultures in amounts which were minimal for eliminating the growth inhibition by hydroxyproline markedly reduced the incorporation of hydroxyproline (Table 3). These results suggest that a certain level of hydroxyproline incorporation must be attained before growth is inhibited.

Specificity studies on prolyl-RNA synthetase from sycamore will be undertaken in our laboratory, together with attempts to determine the effect of hydroxyproline incorporation on protein function. In view of the proposed role of hydroxyproline-rich glycoprotein ("extensin") in providing a network containing labile cross-links in plant cell walls,[16] consideration must be given to the possible effect of hydroxyproline incorporation on the properties of wall glycoprotein. We shall also study the hydroxyproline-deficient glycoprotein which is synthesized in the presence of $\alpha\alpha'$-dipyridyl. For example, the enhanced elongation of wheat coleoptiles grown in the presence of $\alpha\alpha'$-dipyridyl[23] might be related to a reduced hydroxyproline content of the cell wall.

Summary.—The fact that $\alpha\alpha'$-dipyridyl totally inhibited proline hydroxylation in sycamore cells made it possible to demonstrate direct incorporation of hydroxyproline into protein of sycamore cells exposed to a growth-inhibitory concentration of hydroxyproline (0.2 mM). Hydroxyproline was not incorporated when present at a noninhibitory concentration (0.007 mM); the evidence indicates that the hydroxyproline found in protein under these conditions was incorporated as proline and subsequently hydroxylated. When hydroxyproline inhibited the growth of sycamore cells, there was no decrease in the incorporation of proline, nor was there any change in the extent of proline hydroxylation over the period studied. Thus growth inhibition probably stems from the incorporation of hydroxyproline into proteins which are thereby rendered nonfunctional.

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